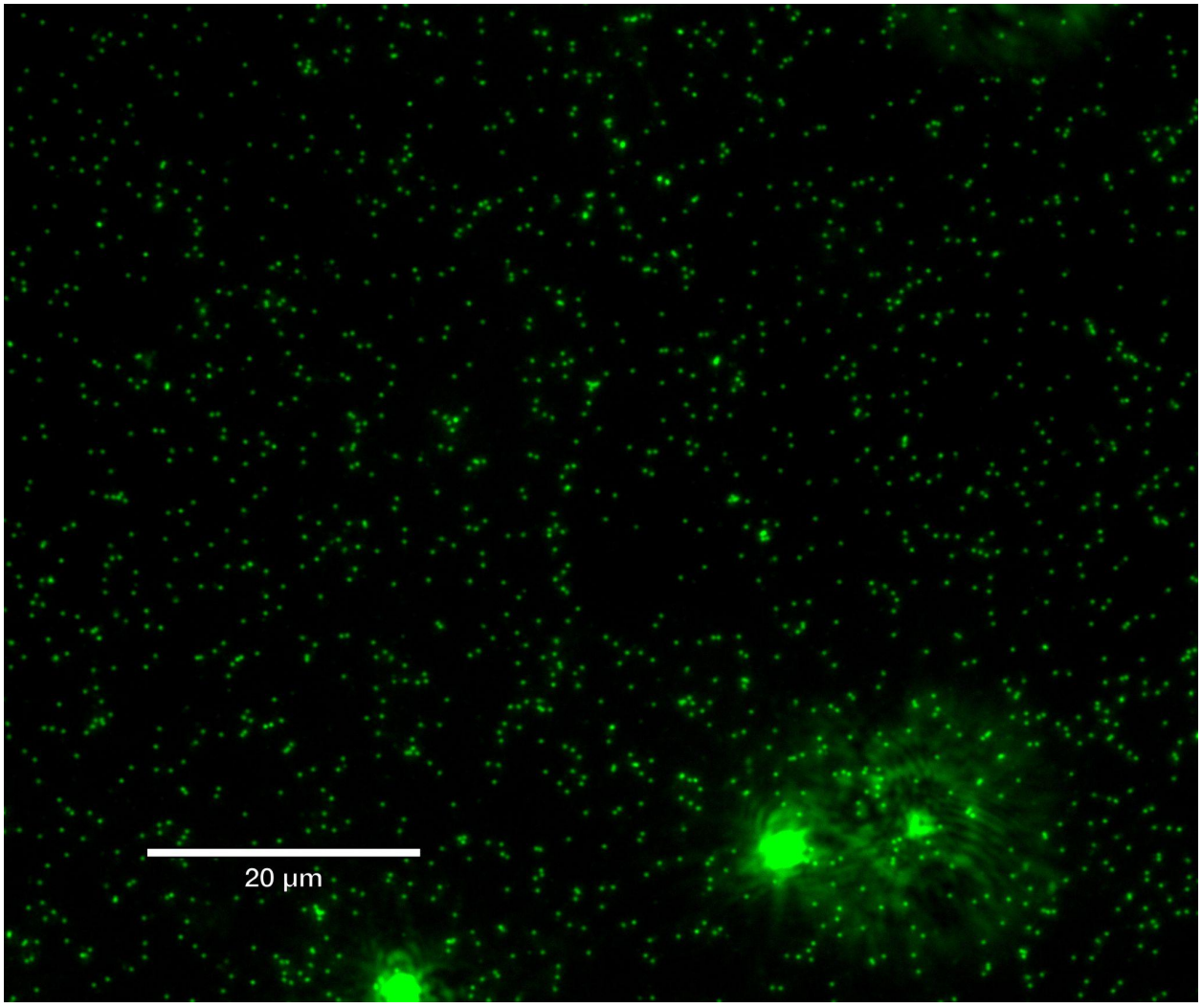
V2 Date: March 20th, 2023
RAW LAB
University of North Carolina at Charlotte

PROTOCOL: Epifluorescence Microscopy for Virus and Bacteria Enumeration



Version 02

March 20th, 2023

University of North Carolina at Charlotte

(Charlotte)

MATERIALS

Consumables

- Whatman grade 1 qualitative filter paper
- 0.65 µm PVDF Durapore membrane filter (hydrophilic)
- 0.22 µm PVDF Durapore membrane filter (hydrophilic)
- 30 kDa MWCO Millipore centrifuge filters (of desired size)
- Low protein binding, nuclease free microcentrifuge tubes (1.5 and 2 ml)
- P1000 pipette tips
- P200 pipette tips
- P10 pipette tips
- 5 ml Eppendorf tubes
- Slides
- Slide covers

Chemicals

- 70% ethanol
- 25% EM grade glutaraldehyde
- Chloroform
- Poly-L-Lysine 0.1% w/v
- SYBR Gold nucleic acid stain
- 10x PBS
- Ascorbic acid
- Microscope immersion oil with a refractive index of 1.515 to 1.517
- EDTA
- Benzonase

Equipment

- P1000 pipette
- P200 pipette
- P10 pipette
- Centrifuge capable of holding 50 ml conical tubes (29 mm diameter; 124 mm length) or wide neck bottles (62 mm diameter; 146 mm length) if using Centricon-70 plus centrifuge filters
- Microcentrifuge
- Vortexer
- Probe sonicator
- Heat block
- Balance
- Microscope equipped with a 100x oil immersion lens and blue excitation light (495 nm)

PERSONAL PROTECTIVE EQUIPMENT AND SAFETY

1. Prepare appropriately with the appropriate PPE: a lab coat, nitrile gloves, goggles, and face shield (for work with glutaraldehyde and chloroform).
2. Clean every work surface with 70% ethanol before and after working. Fixation with glutaraldehyde and chloroform treatment needs to be performed in a fume hood with a face shield.
3. Needles used to remove glutaraldehyde from serum vials need to be disposed of in a sharps container within the fume hood. Never replace the cap on a needle. Once the needle has been used, immediately deposit it into the sharps container.
4. Any excess glutaraldehyde and chloroform must be disposed of into glass waste containers in the fume hood. Solid waste (pipette tips, microcentrifuge tubes, etc.) must also be disposed of into glass waste containers in the hood.

WATER

Step A - Filtration

1. Record the starting volume of water, this will be used for calculations later
2. Filter water through a glass fiber pre filter (Whatman Grade 1 qualitative filter paper)
3. Collect filtrate and filter through a 0.65 µm PVDF filter (Durapore PVDF membrane filter; hydrophilic)
4. Collect filtrate again and filter two times through 0.22 µm PVDF filters (Durapore PVDF membrane filter; hydrophilic)
5. If only filtering samples, proceed to **Step B - Fixation**. Continue this section for optional concentration.
6. Collect filtrate and add to the top portion of 30 kDa MWCO Centricon-70 plus centrifuge filters (Millipore UFC703008)[A1]
7. Centrifuge for 12 minutes at 3500 rpm (if using smaller volume filters adjust speed and time according to manufacturer instructions)
8. After centrifugation, collect the ultrafiltrate from the collection cup below the filter and add more 0.22 µm filtered water to the top. Continue to centrifuge until all water has passed through the filter.
9. After concentrating all the water, attach the retrieval cup to the top of the filter and flip upside down[A2]. Centrifuge for 15 minutes at 3500 rpm.

10. Collect the concentrate from the retrieval cup and pipette into a 2 mL low protein binding nuclease free microcentrifuge tube. Record the volume of concentrate recovered.

11. Dilute concentrate ~3:10 with ultrafiltrate[A3]

12. Add 500 µL of chloroform to the sample.
    a. A lab coat, face and eye protection, and double gloves should be worn whenever working with chloroform, in addition to being performed in a fume hood.
    b. A small glass pasteur pipette must be used when working with chloroform. The pasteur pipette's full volume should be 500 µL.

13. Centrifuge the samples for 5 minutes at 14,000 RPM (standard mini-fuge speed).

14. Use an appropriately sized pipette to carefully pipette sample portion (top part) of the supernatant, making sure to not get any chloroform.

15. Aliquot 92 µL of the diluted concentrate into a 1.5 ml low protein binding nuclease free tube, **proceed to Step B - Fixation.**

Step B - Fixation

Fixation should be done in a fume hood as it requires working with glutaraldehyde Additionally, a lab coat, face and eye protection, and gloves should be worn whenever working with glutaraldehyde.

1. Add 8 µL of EM grade 25% glutaraldehyde to each of the 92 µL samples (final concentration of 2%). Pipette to mix.

2. Allow samples to fix in the dark at 4ºC for 30 minutes

3. OPTIONAL: Freeze at -80ºC until use
    a. If possible, flash freezing using liquid nitrogen should be performed over freezing in a -80ºC freezer. Samples can be stored in the -80ºC freezer until use.
    b. NOTE: This is an optional stopping point. Freezing is not necessary and may cause a decrease in viral counts. If you do not have time to proceed to dyeing and imaging, freeze your samples until use.

Step C - Preparation for EFM and Imaging

1. Thaw fixed samples at room temperature (if applicable). During this time, start heating a heat block to 80ºC. Start heating another heat block to 37ºC.
    a. If working in the RAW lab, use the thermocycler on the EFM setting for fast heating/cooling times.

2. Aliquot 19 µL of each sample into new 1.5 µL low protein binding tubes.

3. Add 1 µL of benzonase to each sample. Pipette to mix.

4. Incubate the samples in a heat block at 37ºC for 30 minutes.
5. Add 4 µL SYBR Gold working stock, pipette to mix.
    a. See **NUCLEIC ACID WORKING STOCK** below for instructions on making working stock.
    b. All work with SYBR Gold should be performed in the dark (or low light) as dye is light sensitive.
6. Incubate in a heat block covered in aluminum foil at 80ºC for 15 minutes.
7. While samples are on the heat block prepare 10% ascorbic acid antifade solution[C1]
    a. Dilute 10x PBS 1:10 by adding 100 µL 10x PBS to 900 µL autoclaved Nanopure water (18.2 MΩ)
        i. Alternatively, 1 mL 1X PBS may be used if available.
    b. Add 0.1 g ascorbic acid and mix thoroughly by vortexing
    c. Allow ascorbic acid to dissolve completely and then filter twice through 0.22 µm syringe filters
        i. Air may have to be pushed through the syringe to get all of the solution out of the filter.
8. Remove from heat block and add 2 µL of ascorbic acid antifade solution, pipette to mix
9. Pipette 10 µL onto a labeled, polylysine prepared slide
    a. See **SLIDE PREPARATION** below for instructions on preparing polylysine slides.
10. Gently cover with a cover slide, avoiding creating air bubbles
11. Image on microscope under FITC blue excitation light (495 nm) with a 100x oil stage
    a. Instructions for microscope use can be found at: Microscopy Use Protocol

Notes

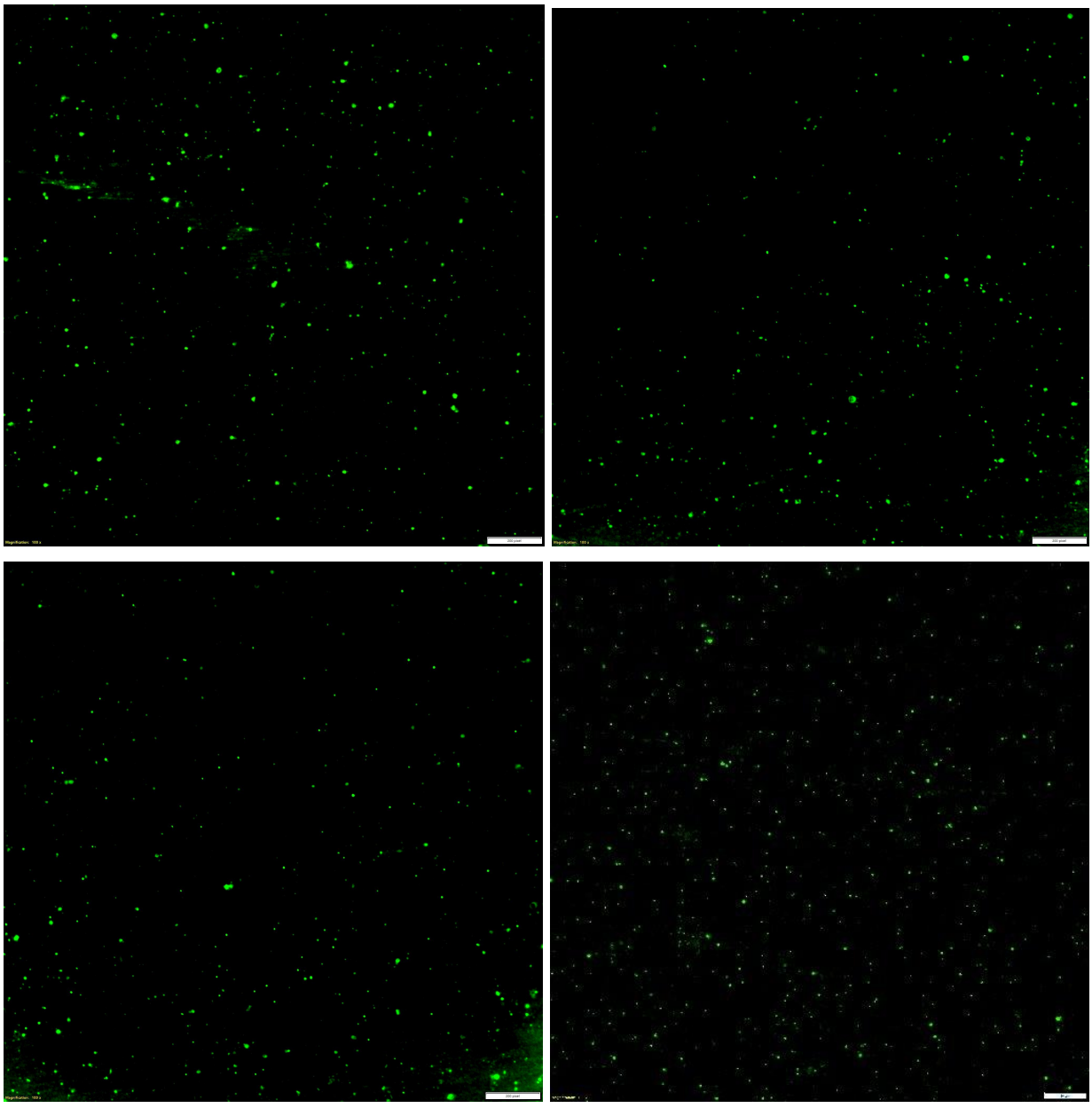
[A1] Centrifuge filters come in a range of volumes (0.5 - 70 mL). These filters can be reused for similar samples. If storing used filters for reuse, after removing concentrate add a small amount of ultrafiltrate or sterile/filtered (0.22 um x2), enough to cover the filter surface, and store at 4ºC.

[A2] Centricon-70 plus filters come with retrieval cups but smaller sizes do not. Smaller sized filters can be removed and flipped upside down into a 15 or 50 mL conical tube and centrifuged to collect concentrate. Amicon ultra-15 filters do not fit into conical tubes. To collect concentrate use a pipette to remove concentrate from the filter area, careful not to puncture the filter.

[A3] Dilution amount can differ depending on the particle density of the sample.

[C1] Ascorbic acid antifade needs to be prepared fresh each time so only prepare a small amount as needed

Examples



MICROBIAL MAT

Step A - Sample Collection

1. Tare a 1.5 µL low protein binding nuclease free tube on a top loading balance
2. Collect approximately 100 mg of mat using a scoopula or similar tool cleaned with 70% ethanol and transfer to the tube. Record the mass of the mat sample

3. Add 100 µL ultrafiltrate from **WATER: Step A - Filtration** to the tube (must use water collected from the same location as the mat sample).

4. Vortex at medium-high speed for 10 seconds.

5. Add 500 µL of chloroform to the sample.
    a. A lab coat, face and eye protection, and double gloves should be worn whenever working with chloroform, in addition to being performed in a fume hood.
    b. A small glass pasteur pipette must be used when working with chloroform. The pasteur pipette's full volume should be 500 µL.

6. Centrifuge the samples for 5 minutes at 14,000 RPM (standard mini-fuge speed).

7. Carefully pipette 92 µL of the top layer of supernatant into a new 1.5 µL low protein binding, nuclease free microcentrifuge tube. **Proceed to Step B - Fixation**.

Step B - Fixation

Fixation should be done in a fume hood as it requires working with glutaraldehyde Additionally, a lab coat, face and eye protection, and gloves should be worn whenever working with glutaraldehyde.

1. Add 8 µL of EM grade 25% glutaraldehyde to each of the 92 µL samples (final concentration of 2%). Pipette to mix.

2. Allow samples to fix in the dark at 4ºC for 30 minutes

3. OPTIONAL: Freeze at -80ºC until use
    a. If possible, flash freezing using liquid nitrogen should be performed over freezing in a -80ºC freezer. Samples can be stored in the -80ºC freezer until use.
    b. NOTE: This is an optional stopping point. Freezing is not necessary and may cause a decrease in viral counts. If you do not have time to proceed to dyeing and imaging, freeze your samples until use.

Step C - Preparation for EFM and Imaging

1. Thaw fixed samples at room temperature (if applicable). During this time, start heating a heat block to 80ºC. Start heating another heat block to 37ºC.
    a. If working in the RAW lab, use the thermocycler on the EFM setting for fast heating/cooling times.

2. Aliquot 20 µL of each sample into new 1.5 µL low protein binding tubes.

3. Add 0.1 mM of EDTA with the tube on ice (4 µL of 0.5 mM EDTA found in liquid chemicals cabinet).

4. Incubate the samples on ice for 15 minutes in the dark.

5. Probe sonicate the samples in three 10 second intervals with 10 seconds between each interval at 50 Hz/40 amp while in an ice bath.
    a. Clean the probe tip with 70% ethanol before and after each sample has been through the entire sonication cycle.
    b. Use a foam tube rack to keep the mouth of the tube out of the water.
    c. Make sure to use a probe sonicator with a microtip to ensure the probe fits in the tube and makes contact with the sample for the duration of the 10 second interval.

6. Aliquot 19 µL of each sample into new 1.5 µL low protein binding tubes.

7. Add 1 µL of benzonase to each sample. Pipette to mix.

8. Incubate the samples in a heat block at 37ºC for 30 minutes.

9. Add 4 µL SYBR Gold working stock, pipette to mix.
    a. See **NUCLEIC ACID WORKING STOCK** below for instructions on making working stock.
    b. All work with SYBR Gold should be performed in the dark (or low light) as dye is light sensitive.

    c. Incubate in a heat block covered in aluminum foil at 80ºC for 15 minutes..

10. While samples are on the heat block prepare 10% ascorbic acid antifade solution[C1]
    a. Dilute 10x PBS 1:10 by adding 100 µL 10x PBS to 900 µL autoclaved Nanopure water (18.2 MΩ)
        i. Alternatively, 1 mL 1X PBS may be used if available.
    b. Add 0.1 g ascorbic acid and mix thoroughly by vortexing
    c. Allow ascorbic acid to dissolve completely and then filter twice through 0.22 µm syringe filters
        i. Air may have to be pushed through the syringe to get all of the solution out of the filter.

11. Remove from heat block and add 2 µL of ascorbic acid antifade solution, pipette to mix

12. Pipette 10 µL onto a labeled, polylysine prepared slide
    a. See **SLIDE PREPARATION** below for instructions on preparing polylysine slides.

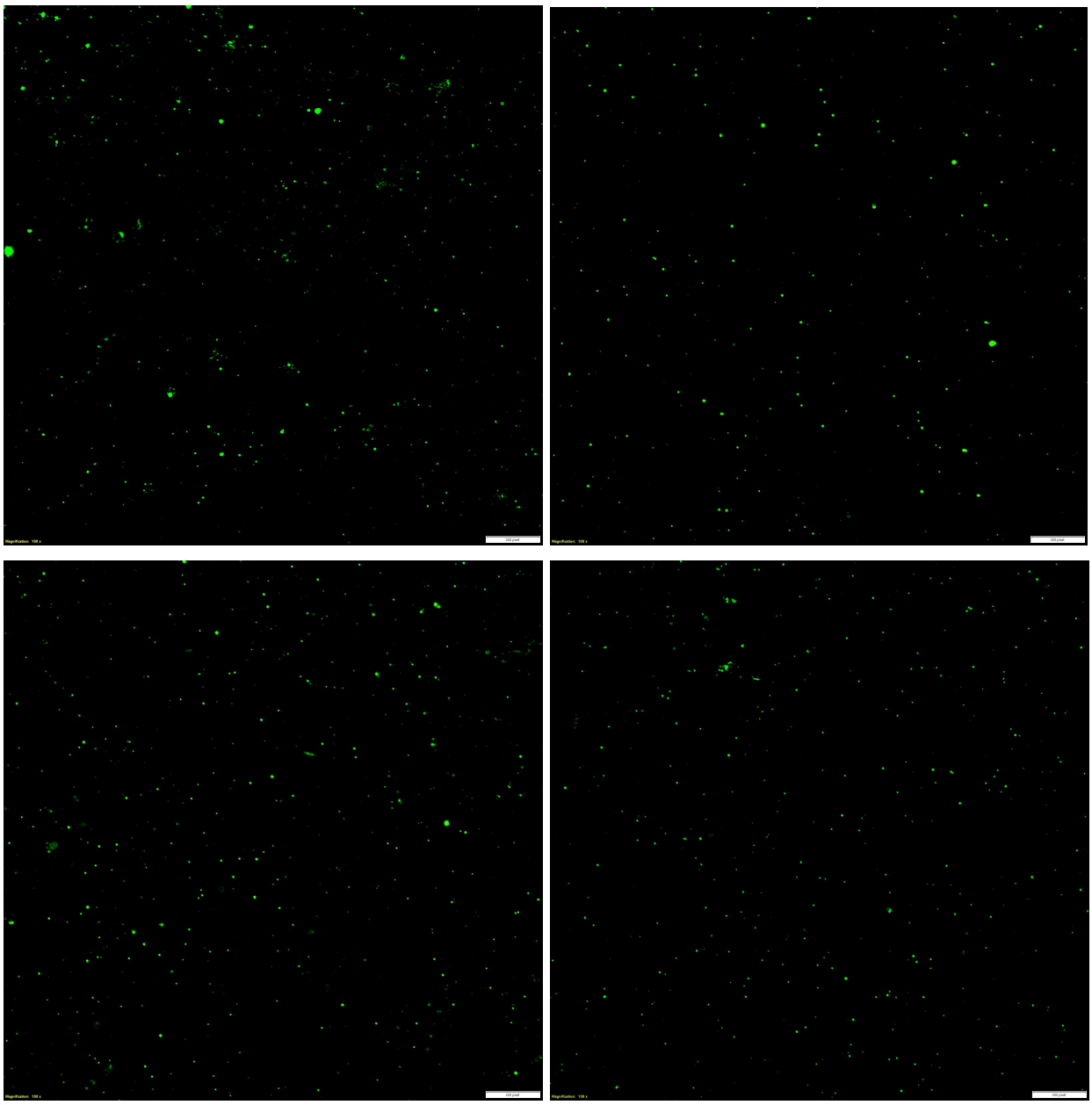
13. Gently cover with a cover slide, avoiding creating air bubbles

14. Image on microscope under FITC blue excitation light (495 nm) with a 100x oil stage
    a. Instructions for microscope use can be found at: Microscopy Use Protocol

Notes

[C1] Ascorbic acid antifade needs to be prepared fresh each time so only prepare a small amount as needed

Examples



EPS (EXOPOLYMERIC SUBSTANCES)

Step A - Sample Collection

1. Tare a 1.5 µL low protein binding nuclease free tube on a top loading balance
2. Collect approximately 100 mg of microbial mat using a scoopula or similar tool cleaned with 70% ethanol and transfer to the tube. Record the mass of the mat sample.
3. Suspend the mat in 900 µL of ultrafiltrate from **WATER: Step A - Filtration** (must use water collected from the same location as the mat sample).
4. Add 500 µL of chloroform to the sample.
    a. A lab coat, face and eye protection, and double gloves should be worn whenever working with chloroform, in addition to being performed in a fume hood.
    b. A small glass pasteur pipette must be used when working with chloroform. The pasteur pipette's full volume should be 500 µL.
5. Centrifuge the samples for 5 minutes at 14,000 RPM (standard mini-fuge speed).
6. After centrifugation, a gelatinous layer should be visible between the bulk mat material collected at the bottom and the overlying supernatant
7. Carefully pipette 92 µL of this layer into a new 1.5 µL low protein binding, nuclease free microcentrifuge tube. **Proceed to Step B - Fixation**

Step B - Fixation

Fixation should be done in a fume hood as it requires working with glutaraldehyde Additionally, a lab coat, face and eye protection, and gloves should be worn whenever working with glutaraldehyde.

1. Add 8 µL of EM grade 25% glutaraldehyde to each of the 92 µL samples (final concentration of 2%). Pipette to mix.
2. Allow samples to fix in the dark at 4ºC for 30 minutes
3. OPTIONAL: Freeze at -80ºC until use
    a. If possible, flash freezing using liquid nitrogen should be performed over freezing in a -80ºC freezer. Samples can be stored in the -80ºC freezer until use.
    b. NOTE: This is an optional stopping point. Freezing is not necessary and may cause a decrease in viral counts. If you do not have time to proceed to dyeing and imaging, freeze your samples until use.

Step C - Preparation for EFM and Imaging

1. Thaw fixed samples at room temperature (if applicable). During this time, start heating a heat block to 80ºC. Start heating another heat block to 37ºC.
    a. If working in the RAW lab, use the thermocycler for fast heating/cooling times

2. Aliquot 20 µL of each sample into new 1.5 µL low protein binding tubes.
3. Add 0.1 mM of EDTA with the tube on ice (4 µL of 0.5 mM EDTA found in liquid chemicals cabinet).
4. Incubate the samples on ice for 15 minutes in the dark.
5. Probe sonicate the samples in three 10 second intervals with 10 seconds between each interval at 50 Hz/40 amp while in an ice bath.
    a. Clean the probe tip with 70% ethanol before and after each sample has been through the entire sonication cycle.
    b. Use a foam tube rack to keep the mouth of the tube out of the water.
    c. Make sure to use a probe sonicator with a microtip to ensure the probe fits in the tube and makes contact with the sample for the duration of the 10 second interval.
6. Aliquot 19 µL of each sample into new 1.5 µL low protein binding tubes.
7. Add 1 µL of benzonase to each sample. Pipette to mix.
8. Incubate the samples in a heat block at 37ºC for 30 minutes.
9. Add 4 µL SYBR Gold working stock, pipette to mix.
    a. See **NUCLEIC ACID WORKING STOCK** below for instructions on making working stock.
    b. All work with SYBR Gold should be performed in the dark (or low light) as dye is light sensitive.
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    a. Dilute 10x PBS 1:10 by adding 100 µL 10x PBS to 900 µL autoclaved deionized water
        i. Alternatively, 1 mL 1X PBS may be used if available.
    b. Add 0.1 g ascorbic acid and mix thoroughly by vortexing
    c. Allow ascorbic acid to dissolve completely and then filter twice through 0.22 µm syringe filters
12. Remove from heat block and add 2 µL of ascorbic acid antifade solution, pipette to mix
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14. Gently cover with a cover slide, avoiding creating air bubbles
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Notes

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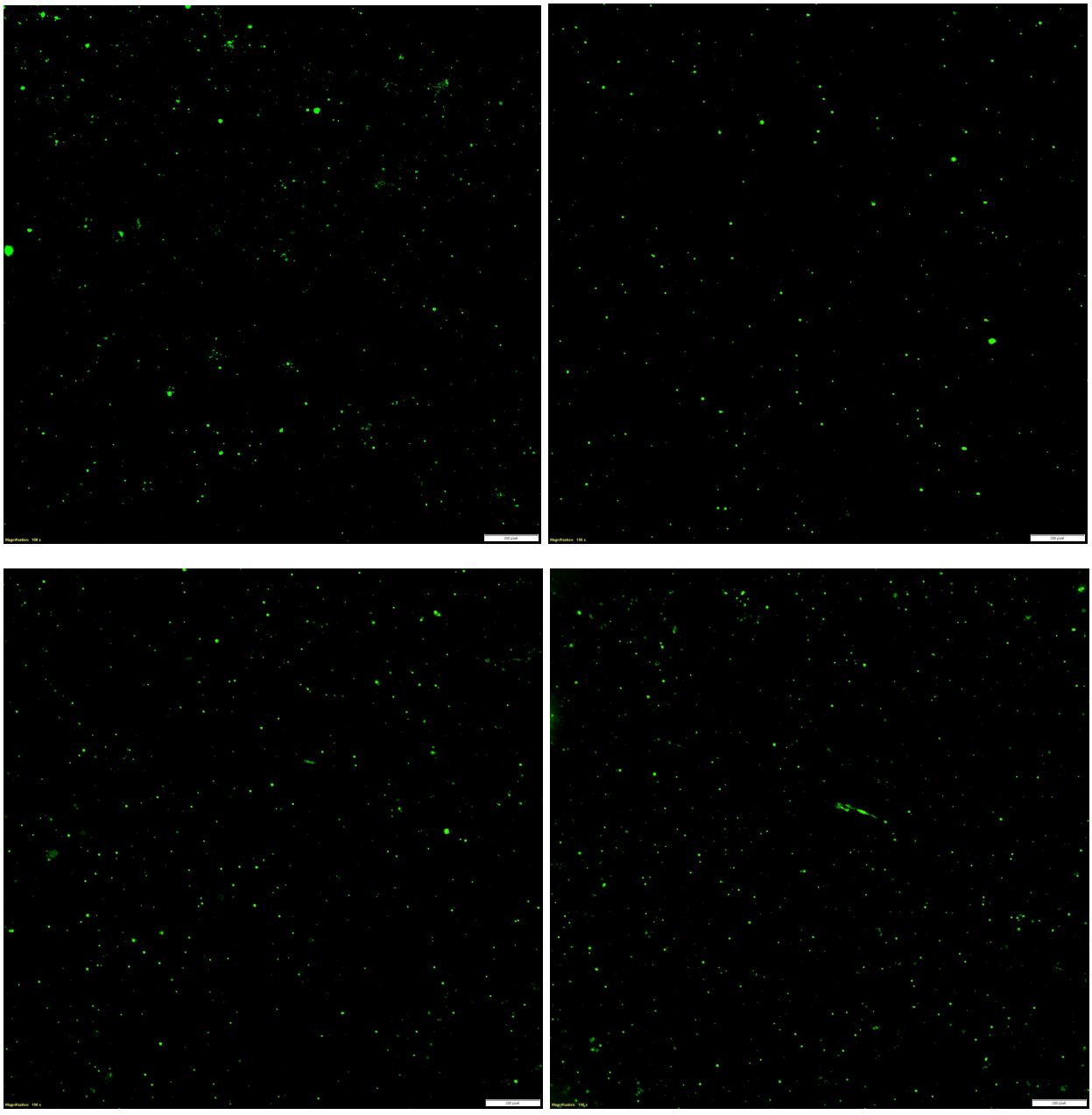
Examples



NUCLEIC ACID WORKING STOCK

The stain is light sensitive so the following steps should be done in the dark. Before preparing working stock, be sure to check if there is already some prepared. Working stocks may be stored in the -20ºC freezer or one working stock at a time may be stored in the 4ºC fridge (found in the black glutaraldehyde box on the top shelf).

1. Thaw commercial stock of SYBR Gold at room temperature, cover with a box or aluminum foil to keep dark.

2. While the commercial stock is thawing, wrap several 1.5 µL low DNA binding tubes in aluminum foil and electrical tape (so the cap is still functional) to protect the working stock from light[1]
    a. Alternatively, black microcentrifuge tubes may be used.

3. Once the commercial stock is thawed, vortex for 10 seconds on medium-high speed, then centrifuge in a microcentrifuge for 5 minutes.

4. Dilute the commercial stock 1:10 with autoclaved and filtered (0.22 µm PVDF membrane filters) molecular biology grade water in a 5 ml eppendorf tube.

5. Filter the working stock through a 0.22 µm syringe filter into a new 5 ml eppendorf tube.

6. Aliquot 250 µL working stock into each of the wrapped tubes.

7. Store working stock at -20ºC.
    a. Working stock that is being used should be stored at 4ºC and can work effectively for about a month but will degrade over time (take note of when working stock is moved to 4º).
    b. Working stock at -20ºC can be stored indefinitely and transferred to 4ºC when ready to use. Avoid freezing and thawing multiple times.

Notes

[1] Multiple working stocks can be prepared at one time and stored at -20ºC until use to avoid multiple freeze/thaws and light exposure of the commercial stock.

SLIDE PREPARATION

Before preparing new slides, be sure to check if there are slides already prepared. These are stored in a small, labeled slide box beside the microscope.

1. Thoroughly clean slides with 70% ethanol and allow slides to dry completely.

2. Prepare a 10% polylysine solution by diluting Poly-L-Lysine 0.01% w/v 1:10 in autoclaved Nanopure water (18.2 MΩ) (using plastic pipette tips and a plastic container).
3. Soak slides in the polylysine solution in a plastic container for 5 minutes (increasing time will not improve performance).
4. Dry slides in a drying oven at 60^{o}C for one hour or overnight at room temperature. Once dried slides can be stored in a plastic slide box at room temperature until use.